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An avian, oncogenic retrovirus replicates *in vivo* in more than 50% of CD4$^+$ and CD8$^+$ T lymphocytes from an endangered grouse

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ABSTRACT

Reoccurring infection of reticuloendotheliosis virus (REV), an avian oncogenic retrovirus, has been a major obstacle in attempts to breed and release an endangered grouse, the Attwater's prairie chicken (*Tympanicus cupido attwateri*). REV infection of these birds in breeding facilities was found to result in significant decreases in the CD4$^+$ and increases in the CD8$^+$ lymphocyte populations, although experimental infection of birds resulted in only increases in the CD8$^+$ lymphocytes. Because our indirect immunofluorescent assay readily detected infection of both CD4$^+$ and CD8$^+$ lymphocytes, a triple labeling flow cytometric procedure was developed to quantify the individual lymphocytes infected *in vivo* with REV. Lymphocytes were gated with a biotinylated pan-leukocyte marker bound to streptavidin R-PE-Cy5. Chicken CD4 or CD8 specific mouse MAb directly labeled with R-PE identified the phenotype and with permeabilizing of cells, infection was indirectly labeled with rabbit IgG specific for the REV *gag* polypeptide and FITC conjugated goat anti-rabbit antibody. More than 50% of the total lymphocytes and of the total CD4$^+$ or CD8$^+$ lymphocytes supported *in vivo* viral expression in all infected birds examined. Remarkably, this level of infection was detected in the absence of visible clinical signs of illness.

Introduction

In 1967, the Attwater's prairie chicken (APC; *Tympanicus cupido attwateri*), a prairie grouse, was placed on the Federal Endangered Species List (Ehrlich, 1992; Johnsgard, 1983). The population decline was mostly due to habitat loss through agricultural and urban development. Beginning in the early 1990s, captive breeding efforts were implemented in Texas for the APC in an effort to prevent extinction, which was the ultimate fate of the heath hen (*Tympanicus cupido cupido*) in the early 1900s (Cokinos, 2000; Drew et al., 1998; Gross, 1928). Despite success in the captive propagation programs, problems have been encountered while attempting to rear and breed APC in captivity. A major obstacle to breeding efforts has been the recurring emergence of infection with an oncogenic retrovirus, reticuloendotheliosis virus (REV) (Drew et al., 1998).

REV are a group of viruses in the family *Retroviridae*, specifically gammaretroviruses in the same genus as mammalian C-type retroviruses (Coffin, 1996). The first documented case of REV infection in prairie chickens (APC and greater prairie chickens [GPC]) occurred in 1993 in the captive breeding flock at the Small Upland-bird Research Facility (SURF), Texas A&M University. The REV group has been associated with acute cell neoplasia, runting disease, and chronic cell neoplasia of lymphoid and other tissues (Fadly et al., 2008; personal observations).

REV strains include REV-T (replication defective and tumorogenic), REV-A (REV-T helper, replication competent), spleen necrosis virus (SNV), chick syncytial virus (CSV), duck infectious anemia virus (DIAV) and other isolates obtained from turkeys, chickens, ducks, pheasants, geese and prairie chickens (Fadly et al., 2008). REV isolates from domestic fowl have been reported in Australia, South America, Europe, and Asia, as well as from ducks in Australia and the U.S., and from pheasants in Hungary. Additionally, REV antibodies have been detected in ostriches in Zimbabwe (Ritchie, 1995). REV has been isolated from birds at every APC captive breeding colony (personal observations). It has been proposed that all REV strains constitute a single serotype. Due to minor, but distinct, differences in neutralization titers, antigenic subtypes have been suggested (Chen et al., 1987). The U.S.A. strains do seem to represent a common genotype with 93 to 99% homology of isolates from chickens, grouse, and ducks during a time frame of 40 years (Bohls et al., 2006b). The host reservoir, prevalence of REV in nature, and the origin of the virus infecting prairie chickens have not yet been determined, although a Texas prairie chicken isolate was 99% identical to an isolate from a domestic chicken in Texas (Bohls et al., 2006b; Barbosa et al., 2007). The first

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prairie chickens identified as infected experienced high morbidity that included wasting and mortality (personal observations; Drew et al., 1998).

REV has been suggested to be immunosuppressive. However, little is known about the interaction of the virus with the avian immune system. Currently, nothing is known of the cellular immune system or the pathogenesis of REV in prairie chickens, including the role of cellular immunity in controlling viral infection. Critical to the avian immune response to viral infection, chicken T lymphocytes have been shown to control antigen specific control of viral infection (Pei et al., 2003; Seo and Collisson, 1997; Seo et al., 2000; Seo et al., 1997). The major obstacle in studying immunity in grouse is the absence of species specific reagents. In order to examine the interactions of REV with T lymphocytes, chicken reagents have been identified that cross-reacted with prairie chicken lymphocytes (Bohls et al., 2006c). A rabbit antiserum specific for recombinant REV-*gag* proteins was used in the current study to identify the subpopulations of lymphocytes infected *in vivo* with REV. Theses studies indicate that more than 50 % of lymphocytes from infected birds support *in vivo* REV replication.

Results

PCR confirms infection in prairie chickens

Nested PCR is highly sensitive and was used to detect virus as early as two weeks after infection. Single step PCR amplification did not consistenly show positive results. Previous work in our laboratory showed detection of virus in all birds regardless of dose of infection two weeks post infection when using the nested PCR (Bohls et al., 2006a).

Flow cytometric analyses identified decreases in CD4$^+$ and CD8$^+$ lymphocyte numbers in naturally infected birds

Flow cytometry with single labeling was used to determine the percentage of peripheral blood mononuclear cells (PBMC), with either the CD4$^+$ or CD8$^+$ phenotype, collected from four naturally infected or 34 uninfected birds. The percent of CD4$^+$ lymphocytes from infected prairie chickens was significantly decreased compared to the same population from uninfected birds (Table 1, $P<0.01$). Whereas the CD4$^+$/CD8$^+$ lymphocyte ratios were typically lower in infected birds as compared with uninfected birds, the percentage of CD8$^+$ lymphocytes of one infected APC (B32) also was decreased, resulting in a normal ratio. Whereas the other birds appeared clinically healthy, this bird was wasting and clinically ill at the time of collection, and died within a month of sample collection. Similar decreases were observed in the CD4$^+$ populations when the total numbers of lymphocytes were calculated by volume of blood. The mean numbers of CD4$^+$ cells from two infected Attwater's prairie chickens were 1340 and 2860/μl of blood compared with 4277 ± 341.38 CD4$^+$ cells/μl of blood collected from uninfected birds while the CD8$^+$ cells/μl of blood were within the range of 26 uninfected Attwater's prairie chickens (1858 ± 1147).

Table 1
Flow cytometric analyses of lymphocytes from Attwater's and Attwater's/greater hybrid prairie chickens suggests REV infection decreases CD4$^+$ lymphocyte numbers

Birds	Lymphocyte phenotype		
	CD4$^+$	CD8$^+$	CD4$^+$/CD8$^+$
Uninfected			
APC (28)[a]	6.65 ± 2.49[b]	2.99 ± 1.06	2.24 ± 0.94
GPC/APC hybrids (6)	8.89 ± 3.44	2.97 ± 0.98	2.99 ± 0.75
Naturally infected			
R100 (APC)	4.35	3.27	1.32
B43 (APC)	2.53	2.05	1.23
B32 (APC)	3.26	1.26	2.59[c]
B243 (hybrid)	2.64	2.06	1.28

[a] Number of birds in the group.
[b] Mean and standard error.
[c] Reflects a drop in both CD4$^+$ and CD8$^+$ lymphocytes. This bird was clinically ill.

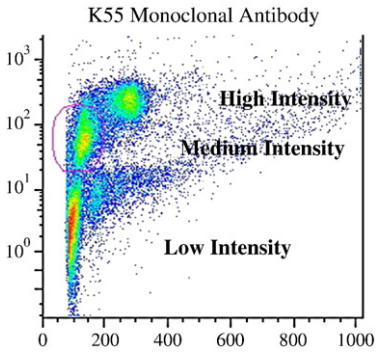

Fig. 1. Flow cytometric analyses after labeling prairie chicken PBMC with the K55 pan-leukocyte MAb differentiated three populations of cells by fluorescence intensity. The lymphocytes are represented in the medium gated population.

The use of flow cytometry with single antibody labeling to evaluate lymphocyte phenotypes in birds is complicated by large numbers of nucleated thrombocytes. In order to eliminate the possibility of thrombocyte associated skewing of results, PBMC from 4 naturally infected and 5 uninfected APC were dual-labeled with K55, a pan leukocyte specific monoclonal antibody (MAb), and either chicken CD4 or CD8 specific MAb (Chung et al., 1991; Bohls et al., 2006c). Cells with the CD4 and CD8 surface antigens were analyzed by gating on lymphocytes, which represented the cell fraction with medium intensity of K55-labeled fluorescence (Fig. 1). The mean of CD4$^+$ cells within the lymphocyte population from REV field infected birds was statistically decreased ($P<0.03$) compared to the mean of the REV negative prairie chickens (Fig. 2). Not observed with the single-labeled technique, a statistically significant increase ($P<0.025$) in the mean of the CD8$^+$ lymphocyte populations was identified for the naturally infected as compared with the uninfected birds.

CD8$^+$ lymphocyte populations increased following experimental REV infection

Field infected birds could be exposed to any number of environmental agents that could be potential co-factors of pathogenesis and

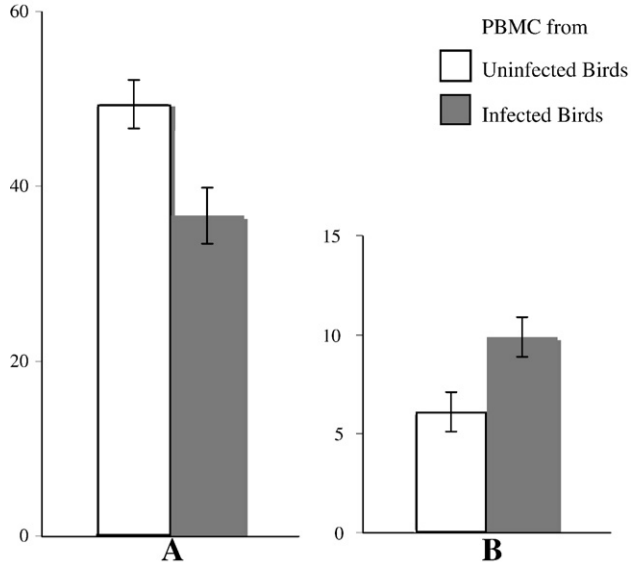

Fig. 2. Using dual labeling to gate lymphocyte populations, natural, chronic infection with REV correlated with a decrease in the population of prairie chicken CD4$^+$ lymphocytes (A) and an increase in the CD8$^+$ lymphocyte population (B).

also could affect lymphocyte numbers. Because of their endangered status, the APC could not be used for experimental infection. Therefore, four adult APC/GPC hybrid birds, bred and housed in a BSL2 environment, were inoculated i.v. with $TCID_{50}$ 5000 as previously described (Bohls et al., 2006a) with a prairie chicken REV isolate (R92) or with PBS alone in order to evaluate changes in lymphocyte populations following experimental infection. Four uninfected APC/GPC hybrids were used as controls. Using the K55 MAb to identify the lymphocyte population, percentages were determined with either the CD4 or CD8 phenotype for lymphocytes from birds 3–5 months after infection when all birds were determined by PCR to be positive for REV. Surprisingly, the mean of the $CD4^+$ population of these chronically infected birds was similar to the PBS inoculated controls. However, the percent mean of $CD8^+$ lymphocytes from infected birds was significantly increased ($P<0.03$) compared to that from the uninfected group (Fig. 3).

Indirect immunofluorescent assays (IFA) identified REV replication in both $CD4^+$ and $CD8^+$ PBMC following in vivo infection

Because fluctuations in both $CD4^+$ and $CD8^+$ lymphocyte populations could be identified following REV infection, it was of interest to determine the potential for the virus to affect cell numbers by direct infection. In order to examine viral infection, purified recombinant REV *gag* polypeptide, expressed with a histidine tag and purified with a nickel column, was used to generate polyclonal antibody in rabbits. The monospecific, polyclonal rabbit anti-sera were shown to react with the recombinant REV *gag* polypeptide in western blots (Fig. 4). PBMC prepared from APC/GPC hybrid birds that were positive for REV and uninfected controls were treated for IFA with Texas red labeled polyclonal anti-REV (*gag* polypeptide) antibody (Figs. 5B, and D). REV replication was negligible in the uninfected birds (Figs. 5A, C). $CD4^+$ (Figs. 5A, B) and $CD8^+$ (Figs. 5C, D) FITC-labeled (green) cells were readily identified in PBMC. A number of PBMC that were both infected and positively labeled with CD4 or CD8 specific MAbs were identified by the dual-label and are shown as yellow cells by merging the images in the software. (Figs. 5B, D).

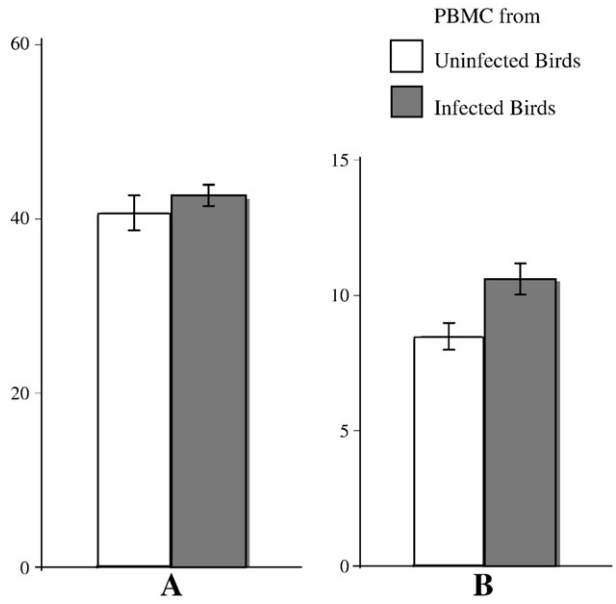

Fig. 3. Using dual labeling to gate lymphocyte populations indicated that while there was no significant impact on the $CD4^+$ lymphocyte numbers (A), experimental, chronic infection with REV did correlate with an increase in the population of prairie chicken $CD8^+$ lymphocytes (B).

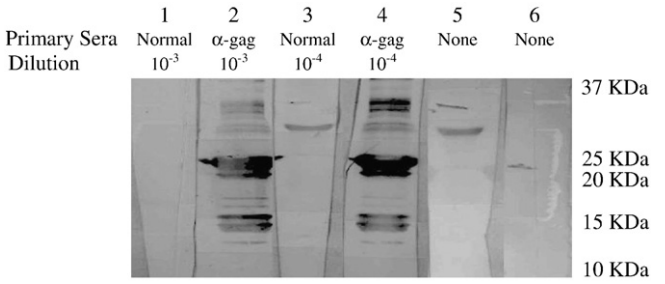

Fig. 4. Western blot of purified REV *gag* polypeptide with polyclonal rabbit anti-*gag* antibody. Lanes 1 and 3 represent *gag* polypeptide reacted with normal rabbit serum (NS). Lanes 2 and 4 represent *gag* protein reacted with rabbit anti-*gag* specific antisera (α-*gag*). Lanes 1 and 2 were blotted with rabbit sera diluted 10^{-3}. Lanes 3 and 4 were blotted with rabbit sera diluted 10^{-4}. Lanes 5 and 6 represent the *gag* protein reacted with the secondary antibody in the absence of primary sera.

Flow cytometric analyses detected viral infection in chicken embryo fibroblasts (CEF)

In order to better define and quantify individual cells infected with REV, a triple label flow cytometry procedure was developed to simultaneously identify infection and $CD4^+$ or $CD8^+$ phenotype of lymphocytes gated following labeling with the pan-leukocyte K55 MAb. REV infection was initially detected with flow cytometry using FITC conjugated rabbit anti-*gag* sera and permeabilized, infected or uninfected CEF (Fig. 4). As compared with 0% of uninfected cells that were antigen positive, 15% of infected CEF were positive for the REV polypeptide (Figs. 6A, B, respectively). No nonspecific binding was seen with rabbit IgG-FITC in either infected or uninfected cells (data not shown). Specificity of infection was confirmed by inhibiting the binding of labeled anti-*gag* antibody using unlabeled antibody at a 20 fold higher concentration (Fig. 6C).

Flow cytometric analyses confirmed that both $CD4^+$ and $CD8^+$ T lymphocytes were readily infected in vivo with REV

PBMC collected from both 5 uninfected and 4 chronically infected APC were permeabilized and triple labeled with rabbit anti-*gag* polypeptide antibody, K55 MAb and either CD4 or CD8 specific MAb. Contour plots of leukocytes identified *in vivo* REV infection of K55 positive cells that also were labeled with CD4 MAb or CD8 MAb. The upper right quadrants of panels in Figs. 7B, D clearly identified $CD4^+$ and $CD8^+$ lymphocytes, respectively, which were also positive for REV *gag* polypeptide. The background values, as shown by the percent of total $CD4^+$ or $CD8^+$ cells collected from the uninfected bird and also expressing *gag* polypeptide, were 1% and 0.4%, respectively (Figs. 7A, C). The infected $CD4^+$ population of bird 118 represented 12.1% of the total lymphocyte population and 77.7% of the total $CD4^+$ cells. Infection was detected in 60.6% of the $CD8^+$ lymphocytes representing 5.5% of the total lymphocyte population from the same chronically infected bird.

Similar rates of *in vivo* infection were detected in three additional chronically infected birds. Using the triple labeling procedure, the cumulative results indicated that between 50 and 64% of the total lymphocytes were infected with REV (Table 2). Although the numbers of infected $CD4^+$ and $CD8^+$ cells could reach 80%, as least one third of the infected lymphocytes were not stained by either of these T lymphocyte markers. The additional infected lymphocytes could represent the B lymphocyte population for which a grouse specific marker has not been identified.

Discussion

In spite of the name, prairie chickens, either GPC or APC, are prairie grouse rather than domestic chickens. The limited availability of

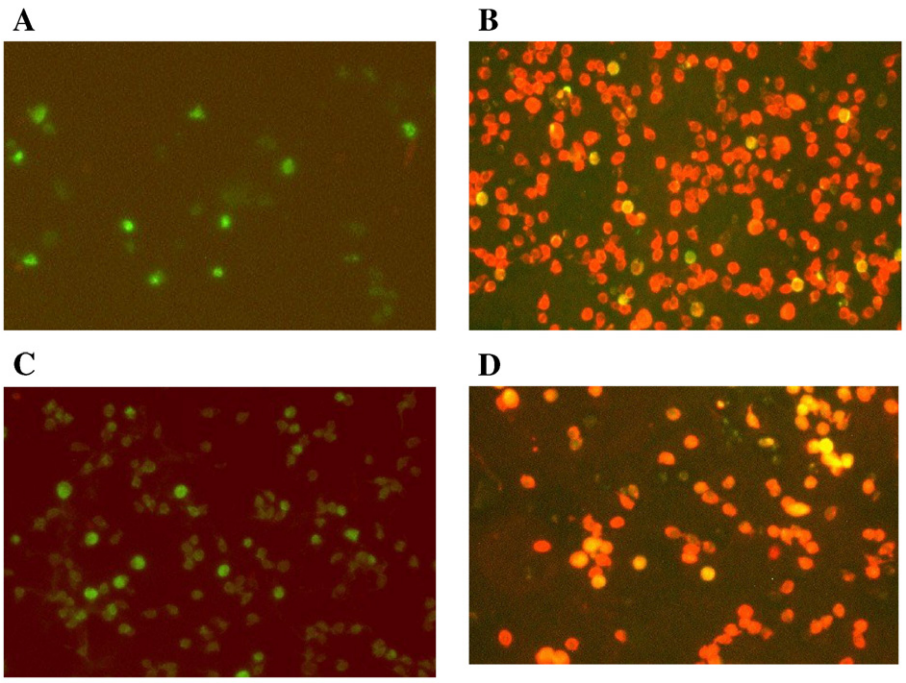

Fig. 5. Indirect immunofluorescent assays identified both $CD4^+$ and $CD8^+$ REV infected cells. Rabbit anti-*gag* polypeptide antibody (Texas red labeled) indicated infection (red cells), and mouse monoclonal antibodies specific for domestic chicken phenotypes (CD4 clone CT4 and CD8 clone 2-293) labeled with Alexa488 identified either $CD4^+$ and $CD8^+$ PBMC as green cells. Yellow cells indicate REV infection of $CD4^+$ or $CD8^+$ cells. Panel A represents $CD4^+$ cells from an uninfected bird; panel B shows labeled $CD4^+$ cells from an infected bird; panel C, labeled $CD8^+$ cells from uninfected bird and panel D, labeled $CD8^+$ cells from an infected bird.

reagents for grouse species has necessitated the use of MAb specific for domestic chicken when possible (Bohls et al., 2006c). Chicken leukocyte and T cell markers had been shown to cross-react with prairie chicken, such that flow cytometry could be used to identify lymphocytes that were expressing either CD4 or CD8 antigens (Bohls et al., 2006c). In the present study, flow cytometry with dual labeled cells was used to identify changes in $CD4^+$ or $CD8^+$ populations from REV infected birds. Experimental and natural infection with REV resulted in an increase in $CD8^+$ lymphocytes, whereas the decrease in $CD4^+$ lymphocyte numbers was observed only in naturally infected birds. Several factors could explain the absence of a decrease in $CD4^+$ cells following experimental infection. Natural infection occurred in birds held outdoors in breeding facilities where they could be exposed to any number of co-factor pathogens, whereas housing for birds used for our experimental infection were held in negative air pressure rooms under specific pathogen free conditions. The maintenance of constant temperature, lighting, and humidity, and absence of other environmental stimuli might have reduced levels of stress. In addition, a known REV isolate, which potentially might have differed in pathogenesis from the naturally infecting strains, was used for REV infection.

In poultry, REV has been described as an immunosuppressive virus and infection is often associated with the presence of lymphomas. The mechanism of immunosuppression has not been identified. Co-infections with REV strains increase pathogenesis after Marek's disease virus infection (Zheng et al., 2007). Suppression of T cell mitogen responses of birds infected with REV was described by Rup et al. (1979) and Walker et al. (1983).

The decrease in $CD4^+$ cells in samples collected from breeding facilities could be identified with both the single and double labeled

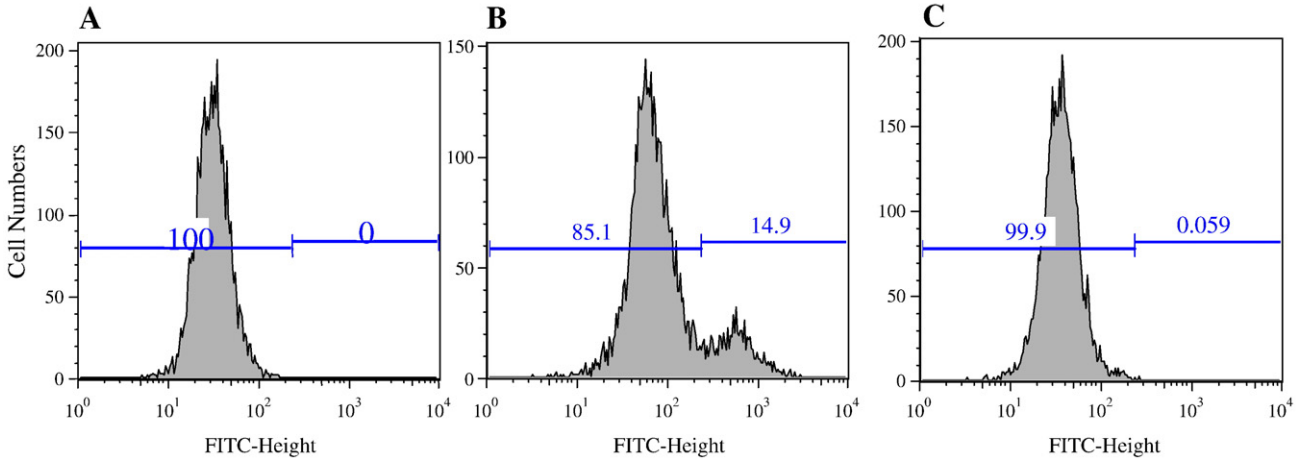

Fig. 6. Histograms evaluating the percentage of cells positive for *gag* antigen in chicken embryo fibroblasts (CEF) infected with REV. Uninfected (A) and infected (B) CEF were labeled with polyclonal rabbit anti-REV *gag*-FITC. Panel C confirms the specificity of the procedure with the blocking of anti-REV *gag*-FITC antibody labeling of infected cells using 20 times the concentration of normal rabbit sera.

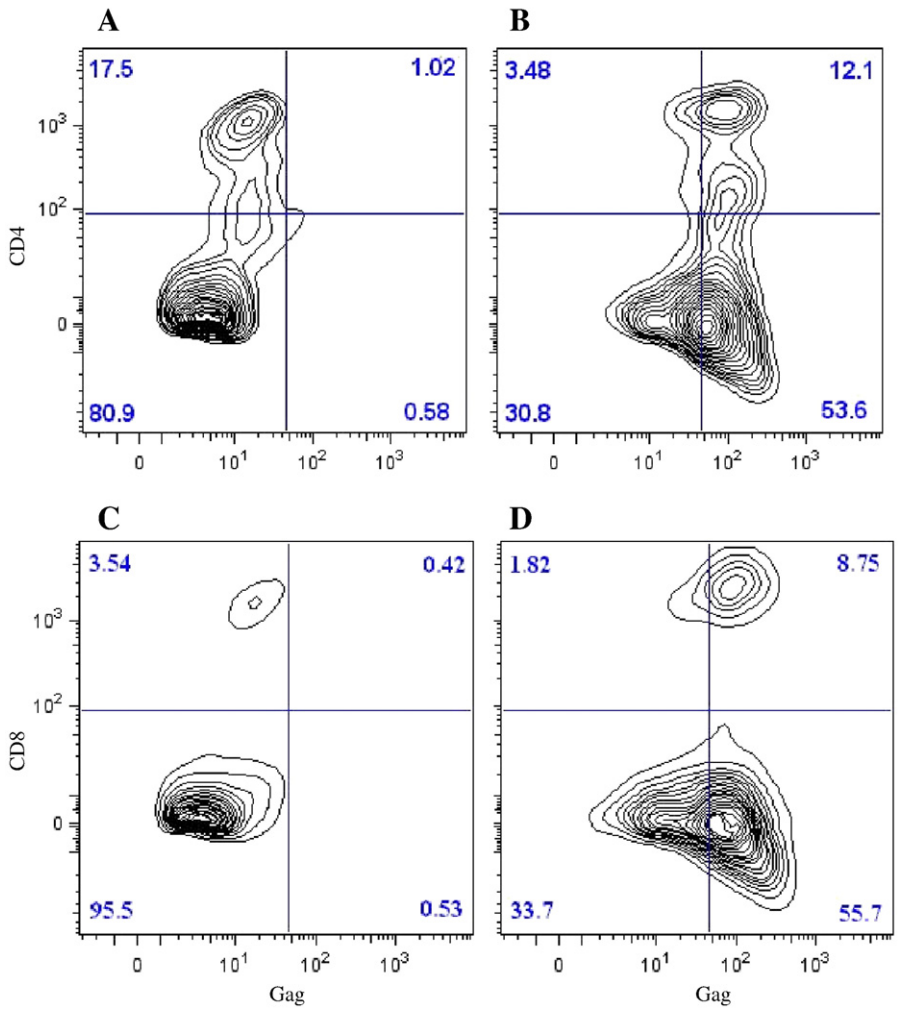

Fig. 7. Lymphocytes from Attwater's prairie chicken PBMC labeled with CD4 (A and B) or CD8 (C and D) MAb and REV *gag* antigen specific antibodies demonstrated infection of both phenotypes. PBMC used for panels A and C were collected from uninfected bird 435 and B and D from REV infected bird 118. The upper right quadrant represents cells positive for both CD4 or CD8 and REV *gag*.

procedures. Whereas the large numbers of thrombocytes would be expected to camouflage significant differences in lymphocyte populations, labeling with the pan leukocyte K55 MAb gated leukocytes into three distinct populations. As shown by Bohls et al. (2006c), the lymphocytes could be identified as labeled with medium intensity of K55 MAb. Using the more sensitive double labeled analyses of lymphocytes, it was possible to identify a significant decrease in $CD4^+$ lymphocytes and a significant increase in $CD8^+$ lymphocytes. However, experimental infection of a prairie chicken with isolate R92 resulted in only increases in the $CD8^+$ populations.

Table 2
Using flow cytometry, lymphocytes from chronically infected birds were gated with the K55 pan leukocyte MAb and evaluated for expression of REV *gag* protein and either CD4 or CD8 antigens

Bird #	REV-PCR[a]	% of Lymphocytes *gag*$^+$			% $CD4^+$	% $CD8^+$
			& $CD4^+$	& $CD8^+$	+ for *gag*	+ for *gag*
435	Negative	1.6	1.0	0.4	5.4	10.6
242	Negative	1.1	0.6	0.4	3.8	10.5
59	Positive	64.4	10.1	8.8	55.9	82.8
104	Positive	60.4	5.7	7.0	79.6	84.0
118	Positive	50.7	12.1	5.5	77.7	60.6
433	Positive	53.1	10.1	3.8	60.8	61.0

[a] REV nested PCR of PBMC was used to identify REV positive birds.

Direct infection could functionally affect cells of the immune system and explain the immunosuppressive nature of REV. The alterations in phenotype of lymphocytes could be associated directly with infection or indirectly, as a result of immune stimulation of $CD8^+$ T lymphocytes. A decrease in $CD8^+$ cells in the single bird was associated with wasting, suggesting an association with immunosuppression. Feline leukemia virus, also a gamma retrovirus, productively infects lymphocytes, monocytes and granulocytes during persistent infection (Pepin et al., 2007; Cattori et al., 2008). Similarly, HTLV-1 infection of human lymphocytes has been evaluated in purified pools of CD4 or CD8 lymphocytes, using PCR quantification (Nagai et al., 2001). These studies determined infection after preparing cell suspensions purified by flow cytometry. Flow cytometric analyses of HTLV-1 infection, using dual labeling with phenotype specific MAbs and a Tax specific MAb, demonstrated that 30% of the $CD8^+$ and 20% of the $CD4^+$ cells were infected. However, in these studies cells were cultured prior to *ex vivo* quantification of infection. In our study, anti-REV *gag* antibody was used to identify the numbers of individual cells of each phenotype that were infected *in vivo* with REV. Therefore, we were able to quantify infected cells expressing either CD4 or CD8 antigen, rather than pools of cells.

Clearly, greater than 50% of the prairie chicken lymphocytes, of either the $CD4^+$ or $CD8^+$ populations were infected with REV, expressing enough of the *gag* proteins to be detected both by IFA and by flow cytometry. Even after adjusting for background fluorescence shown in the infection negative controls, 50 to 72% of the T

lymphocytes were infected. Since 33 to 48% of the infected lymphocytes were not labeled with either T cell marker, a large number of B lymphocytes also were potentially supporting viral replication. There is no B-cell marker available to test this hypothesis. REV seems to be promiscuous for leukocytes, in general, because approximately 90% of the K55 gated fractions representing either monocytes or thrombocytes also could support infection in chronically infected birds (unpublished data).

Although birds are known to behaviorally mask illness, no weight loss or other non-behavioral changes associated with illness were observed. It is remarkable that birds appear clinically healthy with such a magnitude of infection. In the absence of clinical illness, necropsy and histopathology evaluations have identified lesions and neoplasia following experimental infection (Bohls et al., 2006c). Although our experience has been that REV infected prairie chickens do eventually succumb to infection, the capacity to replicate for months in the majority of the leukocytes of an active animal would assure prolonged transmission and survival of the virus. Early detection of infection is critical in maintaining flocks free of infected birds that can remain persistently infected for months. The current assay of choice for detection of REV is PCR amplification of either the polymerase gene or the LTR of the integrated provirus (Drew et al. 1998; Bohls et al., 2006a). The ready identification of infection in leukocytes by direct detection of immobilized cells expressing REV antigen, as with IFA or immunoperoxidase staining, may provide the basis for a more sensitive, less expensive assay.

Materials and methods

Preparation of peripheral blood mononuclear cells

REV infected and uninfected APC, GPC and hybrids (APC/GPC) were housed by SURF located at Texas A&M University, College Station, Texas. Blood was collected from prairie chickens by jugular venipuncture. Peripheral blood mononuclear cells (PBMC) were isolated using Histopaque-1077® according to the manufacturer's instructions (Sigma Diagnostics, St. Louis, MO). Briefly, at room temperature, 5 ml Histopaque®-1077 (Sigma Diagnostics, St. Louis, MO) was overlaid with a blood/Alsever's mixture (1:1) and centrifuged for 20 min at 400 ×*g* at room temperature. Following centrifugation, the opaque interface was collected and washed twice at 4 °C with phosphate buffered saline containing 0.2% bovine serum albumin and centrifuged for 10 min at 250 ×*g* at 4 °C. Cell numbers were counted using a hemocytometer and the cell concentration adjusted to a 1×10^6 cells/ml. Whole blood smears were made for leukocyte differential evaluations (Campbell, 1995).

Polymerase chain reaction assay

All blood samples were tested for the presence of REV proviral DNA sequences using a nested PCR assay. DNA was extracted using a DNA Miniprep kit from Sigma (St. Louis, MO) according to manufacturer's protocol. Primers were designed from sequences obtained from the GenBank database. Primer sets were selected from the polymerase region of the REV gene (Bohls et al., 2006b). Outer primer sequences were REV-4006 5′TCCATGGAAAAGACCCGTAG 3′ and REV-5306 5′ CCCAGCCCGAGAATGTGTCTAC 3′, yielding a product of 1306 bp. Nested primer sequences were as follows: REV-4126 5′TACCTTCGGG-CAGGACATAG 3′ and REV-4908 5′TGCGAATACTGAGGGGTTTC 3′, producing a product of 782 bp. 2 µl of genomic DNA (100 ng) was added to a 25 µl reaction containing 10×PCR reaction buffer (NEB, Boston, MA), 10 mM dNTP mix (Invitrogen, Carlsbad, CA), 10 pmol of each primer and 0.125 µl Taq Polymerase (NEB, Boston, MA) and ddH_2O. Samples were run in a MyCycler (Biorad, Hercules, California) under the following conditions: First round PCR, 2 min denaturation at 95 °C followed by 35 cycles at 95 °C for 20 s, 55 °C for 30 s and 72 °C for 90 s. The products were diluted 10 fold with water to reduce background signals in the gel electrophoresis. 2 µl of the diluted product was added to the same reaction mix as in the first round PCR. Second round PCR, 2 m denaturation at 95 °C was followed by 35 cycles at 95 °C for 20 s, 55 °C for 30 s and 72 °C for 60 s. Samples were run on 1% agarose gels to confirm presence of virus.

Preparation of REV gag polypeptide specific antibody

An REV-*gag* 633 base pair sequence was amplified by PCR using forward primer 868, CGGAGTACAGGTCACTGACGAGCG, and the reverse primer GGGCGGGAGAACCCTGAC. According to manufacturer's protocols, the cDNA PCR products were cloned and ligated into the pET Blue-2 expression vector plasmid containing the sequences for a histidine tag (Novagen, Madison, WI). The cDNA was sequenced and used for transfection as previously described (Bohls et al., 2006b). The histidine fusion-*gag* polypeptide was generated in *Escherichia coli* and purified using nickel-nitrilotriacetic acid beads (Qiagen, Valencia, CA). The recombinant polypeptide was used to hyperimmunize rabbits for antibody production (Sargent, Ramona, CA). Rabbit IgG was purified from sera using a HiTrap protein G HP column (Amersham Pharmacia, Uppsala, Sweden), excess biotin was removed with a PD-10 desalting column (Amersham Pharmacia, Uppsala, Sweden).

SDS-PAGE and western blot analysis

SDS-PAGE

Samples were mixed with 2× Laemmli sample buffer and heated at 95 °C for 5 min prior to SDS-PAGE. SDS-PAGE was run in a mini-Protean Electrophoretic Apparatus (BioRad Laboratories, Hercules, CA, 94547) on a 12.5% Tris-glycine (acrylamide) gel with a 4% stacking gel in 1× SDS-PAGE running buffer (25 mM Tris; 250 mM glycine, pH 8.3; 0.1% SDS) at 150 V for 45 min. Kaleidoscope high molecular weight protein standards were used for size comparisons (BioRad Laboratories, Hercules, CA, 94547). Gels were stained with 0.25% Coomassie Blue R (Sigma, St. Louis, MO 63178) in 50% methanol and 10% acetic acid. Gels were destained with 50% methanol and 12.5% acetic acid and then stored in water to allow complete rehydration. Duplicate gels were run for staining and western blot analysis.

Western blot

Gels were sandwiched with a piece of nitrocellulose between two pieces of Whatman 3 mm filter paper, placed in a western blot cassette and submerged in transfer buffer (6 g Tris-base/L; 28.8 g glycine/L; 0.1 g SDS/L; and 200 ml methanol/L) in the electrophoresis apparatus. The transfer was conducted at 65 V for 1 h. Following transfer, proteins were visualized with Ponceau S (BioRad Laboratories, Hercules, CA) and marked with a pencil for future reference. Lanes were cut into individual strips for exposure to antibody. Rabbit anti-*gag* western: The nitrocellulose was blocked overnight in 3% bovine serum albumin (BSA) in tris-buffered saline (TBS). Following blocking the nitrocellulose was washed 4× in TBS-T (50 mM Tris–HCl pH7.5, 200 mM NaCl and 0.02% Tween 20) and 1× with TBS (50 mM Tris–HCl pH7.5 and 200 mM NaCl). Rabbit anti-*gag* sera were diluted in TBS and 1 ml of each dilution was incubated with nitrocellulose strips for 1 h at room temperature. Secondary antibody (goat anti-rabbit) conjugated with alkaline phosphatase was diluted in TBS at 1:2500 and 1 ml of the dilution was incubated with each strip of nitrocellulose at room temperature for 1 h. Strips were washed as described above before developing each strip in 1 ml of NBT/BCIP (KPL, Gaithersburg, MD) for 10 min. The development reaction was stopped with water.

Flow cytometry

Prior to labeling antibody, nonspecific binding by cellular Fc receptors was blocked with normal sera (Li et al., 2000). A final

concentration of 2 mg/ml of normal goat IgG (Sigma Diagnostics, St. Louis, MO) was incubated with 1×10^6 cells/ml of PBMC for 10 m at 4 °C. Aliquots of 50 ml of 10^6 cells/ml were incubated with each antibody diluted in PBA. Biotinylated K55 (diluted 1:100) and R-PE labeled CD4 or CD8 (Southern Biotech, Birmingham, AL), diluted 1:50, were added simultaneously and incubated for 30 min on ice. Cells were then washed with PBA. Streptavidin bound to R-PE-Cy5 (0.2 mg/ml stock) was added to the cells to bind the biotinylated K55 at a dilution of 1:75. Cells were again incubated for 30 min and washed twice with PBA before resuspending in 200 ml 1% paraformaldehyde and incubating for 1 h at 4 °C. Following paraformaldehyde fixation, cells were washed twice with PBA, resuspended in 200 ml of PBA, and stored at 4 °C. Controls were included for secondary antibody and normal primary sera.

Intracellular labeling was conducted using the Fix and Perm Cell Permeabilization Kit (Caltag Laboratories, Burlingame, CA). Cells, labeled for both extracellular and intracellular antigens, were labeled with extracellular antibodies as described above, before permeabilizing according to manufacturer's instructions. Permeabilized cells were then labeled with rabbit anti-*gag* antibody (diluted 1:100) before washing with PBA incubating with goat anti-rabbit FITC conjugated antibody (diluted of 1:500).

A flow cytometry analysis was performed within 24 to 48 h of sample processing with a FACSCalibur™ (Becton Dickinson, San Jose, CA) by the Core Flow Cytometry Facility at Texas A&M University (College Station, TX) as previously described (Bohls et al., 2006c).

Indirect immunofluorescence assay

Purified PBMC were dropped onto microscope slides and fixed in a methanol/acetone mixture (vol 1:1) before air drying. Slides were stored at −20 °C. The PBMC were re-hydrated with PBS for 5 min at room temperature. Prior to labeling, the cells were incubated with PBS containing 5% non-fat dried milk for 15 min. The slides were rinsed with PBS and incubated with primary antibodies diluted 1:300 for 30 min. The primary antibodies used were as followed: mouse anti-chicken CD4 (CT4), mouse anti chicken CD8 (2-292) and REV-*gag* (polyclonal from rabbit). Following incubation the slides were rinsed with PBS and incubated with secondary antibodies goat anti-mouse Alexa 488, (Invitrogen, Carlsbad, CA) and goat anti-rabbit Texas Red (Southern Biotech, Birmingham, AL) diluted 1:500 for 30 min in the dark. Cells were rinsed with PBS and analyzed with a fluorescence microscope (Olympus IX70 Inverted Microscope) and imaging software (Spot Imaging Solutions, Diagnostics Instruments Inc., Sterling Heights MI).

Hematology

Whole blood was collected with heparin for determining total white cell counts were determined using granulocyte concentrations per µl volume of blood as a reference using the Unopette Test 3877 (Becton-Dickinson, Rutherford, NJ).

Statistical analysis

Statistical significance was determined using measures of central tendency: mean, median, mode, and range. Means were trimmed when needed, in order to eliminate outliers (10% trimmed mean). Statistical analyses were performed using a Student's *t* test for independent samples and unequal variances. A two-tailed test was performed, with the exception of the comparison of REV infected APC vs. uninfected APC, where a one-tailed test was performed. A *P*-value of less than 0.05 was considered statistically significant. All statistical calculations were made using the computer program Microsoft Excel.

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